J. Biochem. **144,** 357–362 (2008)
doi:10.1093/jb/mvn074

Valproic Acid Inhibits the Growth of Cervical Cancer both *In Vitro* and *In Vivo*

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Received February 18, 2008; accepted May 22, 2008; published online May 31, 2008

Valproic acid (VPA), a well-known anti-convulsant, is currently under extensive evaluation as an anti-cancer agent. It is known to exert its anti-cancer effect mainly by inhibiting the enzyme histone deacetylase I. In our study, we investigated the effects of VPA on cervical cancer both *in vitro* and *in vivo* cancer models. We examined the effects of acute VPA (0, 1.2, 2.4, 5.0 mM) treatment on cell proliferation in cervical cancer cell lines HeLa, SiHa and Ca Ski and histone acetylation, p21 and p53 gene expression in HeLa cell line. We also investigated the effect of chronic VPA administration in tumour xenograft growth studies. Our results show that with acute treatment, VPA can increase the expression of net histone H3 acetylation and up-regulate p21 expression with no effect on p53 expression. Chronic administration of VPA had a net cytostatic effect that resulted in a statistically significant reduction of tumour growth and improved survival advantages in tumour xenografts studies. Furthermore, we also demonstrated that VPA has a direct anti-angiogenic effect in tumour studies and could potentially be a promising candidate for further cervical cancer trails.

Key words: angiogenesis, cervical cancer, p21, p53, valproic acid.

Abbreviations: HDAC, histone deacetylase; HIF-1α, hypoxia-inducible factor 1α; HPV, human papilloma viruses; H3 Ac, histone 3 acetylation; VEGF, vascular endothelial growth factor; VPA, valproic acid.

Carcinoma of the uterine cervix is one of the highest causes of mortality in female cancer patients worldwide, and due to non-existent or inadequate screening, disease is normally detected at late stage. Improved treatment options for this type of malignancy are highly needed (*1*, *2*). Cervical cancer is a virus-induced disease that is caused by the integration of high-risk infecting human papillomaviruses (HPV) in the host genome. For this reason, the carcinogenesis process of cervical cancer is associated to the expression of the viral oncogenic proteins E6 and E7. These proteins are capable of inactivating p53 and pRb, which induces a continuous cell proliferation with the increasing risk of accumulation of DNA damage that eventually leads to cancer (*3*). The approval of vaccine against HPV has proved to be a milestone in prevention of the disease. But it is still very important to have better post-exposure treatment options.

In recent years, Histone Deacetylase (HDAC) inhibitors have emerged as the potential therapeutic agents for multiple human cancers. Among these, valproic acid has proven as one of the most promising anti-cancer drugs. Valproic acid's (VPA) anti-neoplastic activity was discovered in 1997 (*4*). VPA exerts its action mainly by targeting the enzyme HDAC (*5–7*). Valproic acid and its analogues modulate the behaviour of various tumour cells by inducing differentiation and inhibiting cell proliferation, increasing apoptosis and immunogenicity and decreasing angiogenic potency (*8*). VPA was able to induce apoptosis and inhibit proliferation in a variety of different cancer cell lines (*8–17*), while it is anti-apoptotic and cytoprotective effects have been reported in other cells (*9–13*). VPA induces differentiation in a high number of different cancer cell types (*8*, *14*, *15*). Cancer cells metastasize by adhering to endothelial cell to invade surrounding tissues and to migrate to locations remote from the primary tumour (*16*). VPA was shown to inhibit cellular adhesion, invasion and migration by multiple mechanisms (*8*, *14*, *15*, *17*). It has been proven that blood supply is imperative to growth of tumour beyond a certain size (*18*, *19*). VPA was reported to inhibit angiogenesis by direct effects on endothelial cells as well as by effects on cancer cell expression of pro- and anti-angiogenic factors (*8*). These VPA-induced anti-angiogenic *in vivo* effects had been extensively observed in general angiogenesis models but not so much in models of tumour angiogenesis (*10*, *20*). Recently, the anti-angiogenic activity of VPA was confirmed in a tumour angiogenesis model, in orthotopic medulloblastoma xenografts in mice (*21*). Angiogenesis inhibitors have been extensively employed in treatment of gynecological malignancies. In cervical cancer, correlation has been seen between increased angiogenic markers and stage, grade, tumour size and survival (*22*).

VPA has never been tested to treat cervical cancer in a mouse model this far. Because of its multiple mechanisms

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of tumour targeting, we hypothesized that cervical cancer will be rendered more sensitive to VPA treatment. Our *in vitro* study concentrates on evaluation of different tumour markers associated with VPA treatment while the *in vivo* part of the study focuses on effect on tumour size reduction and survival of athymic mice implanted with cervical cancer xenografts by treating them with VPA.

MATERIALS AND METHODS

Cell Culture—Cervical cancer cell lines HeLa, Ca Ski and SiHa was obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. HeLa and SiHa cell lines were cultured in basal medium Dubecco's modified Eagle's medium (DMEM) (Gibco), while Ca Ski cell line was grown in RPMI 1,640 (Gibco) medium. Both growth media were supplemented with 10% newborn calf serum (Invitrogen) and the cell lines were maintained at 37°C in 5% CO_2.

Cell Proliferation Assay—HeLa, Ca Ski and SiHa cell lines were seeded at 1×10^4/well in 96-well culture plates and incubated overnight with basal medium (DMEM or RPMI 1640) containing 10% FBS. The cells were then treated with medium containing VPA (0, 1.2 and 5.0 mM) for 3 days. The fraction of cells surviving after acute VPA treatment was determined using the Cell Proliferation kit I (Roche, Basel, Switzerland) as per manufacturer's recommendation. To determine the proliferation of the viable cells after acute VPA treatment, cell lines were seeded and treated in the same manner as above. After the 3-day VPA treatment, cells were washed with PBS and fresh basal medium, (without VPA) containing 10% FBS, was added. Viable cells were detected using the Cell Proliferation kit I at 24-h intervals for 3 days to calculate cell proliferation.

Western Immunoblotting—Western blot analysis was performed according to standard protocol (*23*). A 70–80% confluent T175 flasks of HeLa cells were treated with medium containing 0, 1.2 and 5.0 mM VPA for 48 and 72 h. The cells were harvested with 0.05% trypsin/0.53 mM EDTA, washed in PBS and resuspended in 100 μl volume of AL Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). The BCA protein assay kit (Bio-Rad, Hercules, CA) was used to determine total protein concentration and purified bovine serum albumin (Sigma) to generate the standard curve. Concentrated proteins were separated on a 12% Tris–HCl polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked for an hour in blocking buffer [100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20] with 5% non-fat dry milk and then incubated with rabbit anti-acetylated histone H3 (Upstate, Charlottesville, VA) overnight followed by anti-rabbit IgG peroxidase conjugate (Sigma) for 1.5 h at room temperature. Immunoreactive bands were detected using the enhanced chemiluminescence plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Monoclonal anti-h-actin in mouse (Sigma) and anti-mouse IgG-peroxidase (Sigma) were used to detect h-actin in the same blots. Anti-Cip1/WAF1/p21 mouse monoclonal IgG (Upstate) and anti-mouse IgG peroxidase (Sigma) were used to test p21 expression. Purified mouse anti-human p53 monoclonal antibody (BD-Bioscience) was used to detect p53 expression.

Implantation of tumour Xenografts in Mice and Treatment Protocol—Six-weeks-old athymic mice were obtained from Shanghai Laboratory Animal Co. Ltd (Shanghai, China) and were maintained in pathogen-free conditions and fed a standard irradiated chow diet. Animals were bilaterally, subcutaneously injected with HeLa cells (2×10^6) in 0.1 ml of Matrigel (Collaborative Biomedical Products, Bedford, MA), forming two tumours per mouse. Mice were distributed randomly and were kept at 25°C room temperature, 12-h light–dark schedule with free access to food and water. When the tumour volume reached 100 mm^3, the animals were randomly divided into two groups. Valproic acid was dissolved in PBS (0.4% w/v) and was filtered by 0.22 micron syringe filter (Millipore). Test group received VPA solution, while control group received PBS in place of drinking water. tumour sizes of individual mice were measured every two days by vernier calipers.

Histochemistry and Immunohistochemistry—Mice were euthanized 2 weeks post treatment and tumour tissues were collected, fixed with 4% formaldehyde, embedded in paraffin and sectioned for haematoxylin and eosin (H&E) staining and immunohistochemical staining for CD31. H&E staining was performed according to standard histological procedures. Necrosis and vascularization in tumour tissue were observed under a light microscope. Vascular structures in tumour were evaluated by immunohistochemical staining of CD31 with rabbit anti-CD31 polyclonal antibody (Boster, P.R. China). Staining for CD31 was performed on sections using their specific primary antibodies and biotinylated goat anti-rabbit secondary antibody (Boster, P.R. China), incubated with horseradish peroxidase (HRP) labelled Streptavidin, visualized with DAB chromogen, counter-stained with hematoxylin and observed with microscope.

Statistical Analysis—All experiments were done in triplicate and plotted with standard errors of the mean. All statistical analyses were performed using Prism 4.0 (GraphPad, Inc.) using windows XP operating system. Statistical comparisons for paired data were analysed by the Student *t*-test for the *in vitro* assays, while Wilcoxon signed rank test and 2-way ANOVA were used to analyse the statistical significance for *in vivo* xenograft models. Statistical significance was defined as $P < 0.05$.

RESULTS

Effect of VPA on Histone H3 Acetylation—To demonstrate that VPA treatment induces the expression of H3 acethylation in cervical cancer, total cellular lystates prepared from HeLa cells treated with different concentration of VPA (0, 1.2 and 5 mM) for 72 h and were subjected to western blot analysis for histone H3 acetylation, as shown in the Fig. 1. Significant increases in acetylated H3 were observed compared to the untreated ones. This effect was shown to be dose dependent where 1.2 mM dose seemed to have maximal effect in HeLa cells after 72 h of treatment. Beta actin was included to

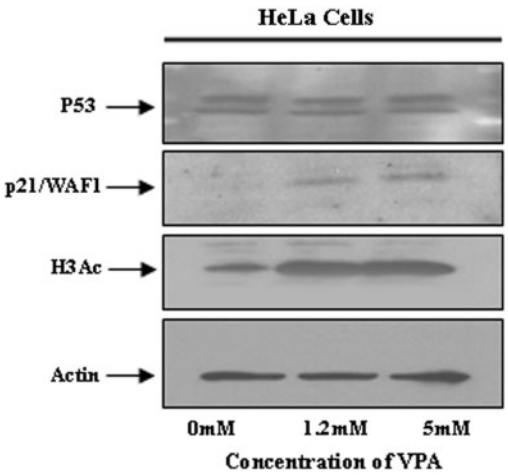

Fig. 1. **Western blot analysis of p53, p21WAF/CIP1 expression and acetylated histone H3 in VPA-treated cells.** HeLa cells were treated with 0, 1.2 and 5.0 mM VPA for 72 h. VPA treatment had no effect on the expression p53. p21WAF/CIP1, on the other hand, showed up-regulation after the treatment. VPA treatment also induces a dose-dependent H3 acetylation in cervical cancer cells. The relative fold increase was determined by scanning densitometry of the western blot.

account for equal loading of the protein sample across the lanes.

VPA Induced p21$^{WAF/CIP1}$ Expression but had no effect on p53—HeLa cells have been known to contain E6 and E7 oncogenes from human papilloma virus 18 (HPV-18). These oncogenes are known to hamper the expression of p53 and pRb. To evaluate if VPA treatment rescues p53 expression from the E6 ubiquitination and restores the normal mitochondrial apoptotic pathway, HeLa cells treated with VPA (0, 1.2 and 5 mM) for 3 days were analysed by western blot analysis. However, no change was detected in the levels of p53 expression (Fig. 1). VPA is also known to arrest the cells at the G_0/G_1 phase by directly modulating the expression of p21/WAF-1 (CDK inhibitor). This protein is also a direct down stream target of the tumour suppressor p53. To investigate if VPA treatment alone has any effect on the p21/Waf-1 expression, we performed western blot analysis on the cell treated with or without VPA (Fig. 1) and found that VPA treatment alone induced the expression of p21/Waf-1 and the effect was not coupled with p53.

VPA Inhibits Cell Proliferation—HeLa, Ca Ski and SiHa cell lines were treated for 72 h with various doses of VPA and then replated (without VPA), and the proliferation was assessed by MTT as indicated in Fig. 2. The data are plotted as fold cell growth, as the various starting points of the different cells varied according to the amount of cell death, which occurred initially from the VPA treatment. All the cell lines showed inhibition of cell proliferation, and this effect was dose-dependent.

VPA Treatment and tumour Sizes—To demonstrate the effect of VPA on the tumour growth, HeLa cells were implanted on both the flanks of athymic mice, when tumour volume reached ~100 mm^3 in volume, animal were randomized in two groups as mentioned in MATERIAL AND METHOD section. During 28 days of treatment,

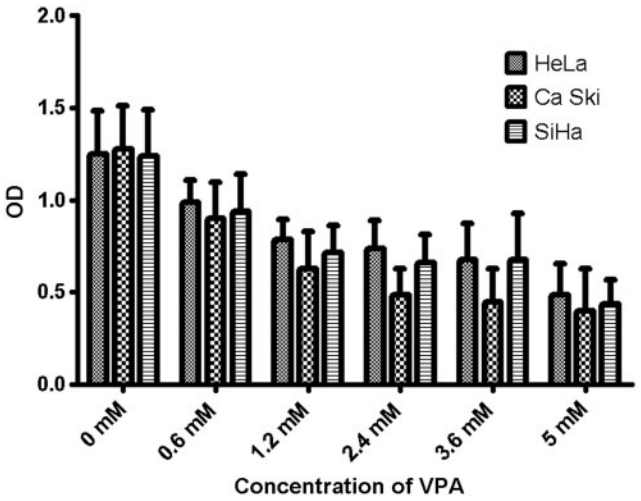

Fig. 2. **Effect of acute VPA treatment on cervical cancer cell lines HeLa, SiHa and Ca Ski, using MTT cell proliferation assay.** All the cell lines were treated with 0, 0.6, 1.2, 2.4, 3.6 and 5.0 mM VPA for 72 h, washed and grown in medium without VPA. Comparison of groups, receiving different concentrations of VPA was done by repeated measures of ANOVA. Results revealed significant differences between treated and untreated groups and the effect was dose-dependent. HeLa and SiHa showed almost equal response to VPA treatment. Ca Ski cell line, on the other hand, was more sensitive to VPA at different doses. There was a significant inhibition of proliferation in all the three cell lines, even at a concentration as low as 1.2 mM. This concentrations falls well within therapeutic range and is quite safe, since the initial signs of toxicity of the drug appear over the 2.5 mM concentration (*in vivo*).

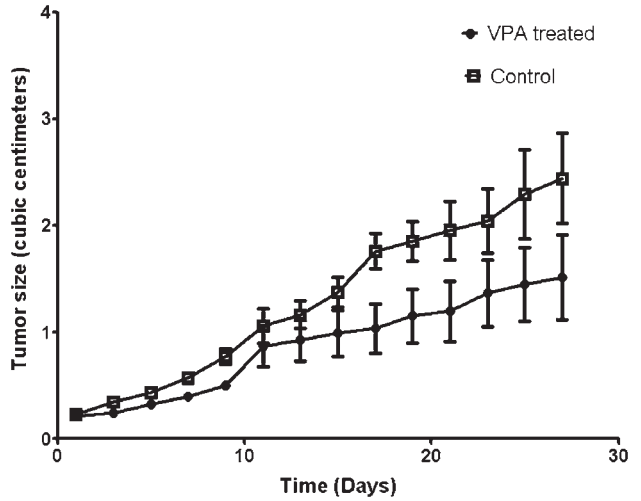

Fig. 3. **The effect of chronic VPA treatment on cervical cancer xenografts.** Tumour xenografts were established in mice by implanting them with HeLa cells on both flanks of the individual mice. Animals in the treatment group received 0.4% VPA in drinking water. Tumour volumes were compared using Wilcoxon rank-sum tests and 2-way ANOVA (*$P < 0.05$).

tumour size of individual mice in both treatment and control groups were measured by vernier caliper every other day. Data was compared using the Wilcoxon rank-sum test and 2-way ANOVA. tumour volumes were plotted against tumour developing time as shown in Fig. 3. Animals treated with VPA showed statistically significant

reduction (upto 30%) in tumour volume compared to that of control group.

Effect of VPA Treatment on Tumour Vasculature—To assess if VPA would inhibit tumour vascularization, mice in both treatment and control arms were euthanized and their tumours were harvested. Fixed tumour xenografts were sectioned by using microtome and fixed on slides. Slides were stained for CD31 expression and counter stained with H&E staining. As shown in the Figs 4A and B, tumour xenografts from treatment group showed lesser blood vessels (Fig. 4B) than that of the control group (Fig. 4A). Difference in vascularization of control and treated arm was quantitatively evaluated by counting the number of small blood vessels at 20× magnification. The number of blood vessels of control versus treated group was plotted (Fig. 4C).

VPA Treatment Significantly Increases Survival in Mice Bearing Xenografts Tumours—The effect of VPA treatment on the survival duration of mice was also evaluated. For that purpose, the mice were monitored daily, during the 28 days of treatment, for the clinical signs of tumour burden. They were removed in case of either natural death or showing extremely heavy tumour burden following the Institutional guidelines for animal care. Survival times were determined by the Kaplan–Meier method using Graphpad Prism software. As shown in Fig. 5 the mean percent survival of VPA treated animals was 66.6% compared to the untreated control which was 33.3% at 28 days.

DISCUSSION

There were about 5,00,000 incident cases of and 2,75,000 deaths due to cervical cancer worldwide in 2002, equivalent to about one-tenth of all deaths in women due to cancer (*24*). The burden of cervical cancer is disproportionately high (>80%) in the developing world (*25*). Not only is cervical cancer the most prevalent and important cancer in women in several developing countries, but also the societal importance of the disease is accentuated even further by the young average age at death, often when women are still raising families. Papanicolaou test (or Pap smear), is the most commonly employed diagnostic test in screening for cervical cancer. But due to the inherent inaccuracies of the procedure and most women forgoing this vital screening, disease is normally detected at a very late stage.

The inhibition of HDACs has emerged as a potential strategy to reverse aberrant epigenetic changes associated with cancer (*26*). Besides, there has been precedence of treatment of cervical carcinoma with HDAC inhibitors and also a phase one study was conducted to evaluate efficacy and safety of VPA (*27*). But beyond the usual HDAC inhibitor activity, the anti-cancer effect of VPA on cervical cancer has never been characterized. VPA has shown great promise in treating different types of cancers, at many levels. Because of its effects on multiple cancer check points, it has the potential of being an excellent conventional chemotherapeutic drug as well as an aid for the other anti-cancer agents, with a considerable margin of safety.

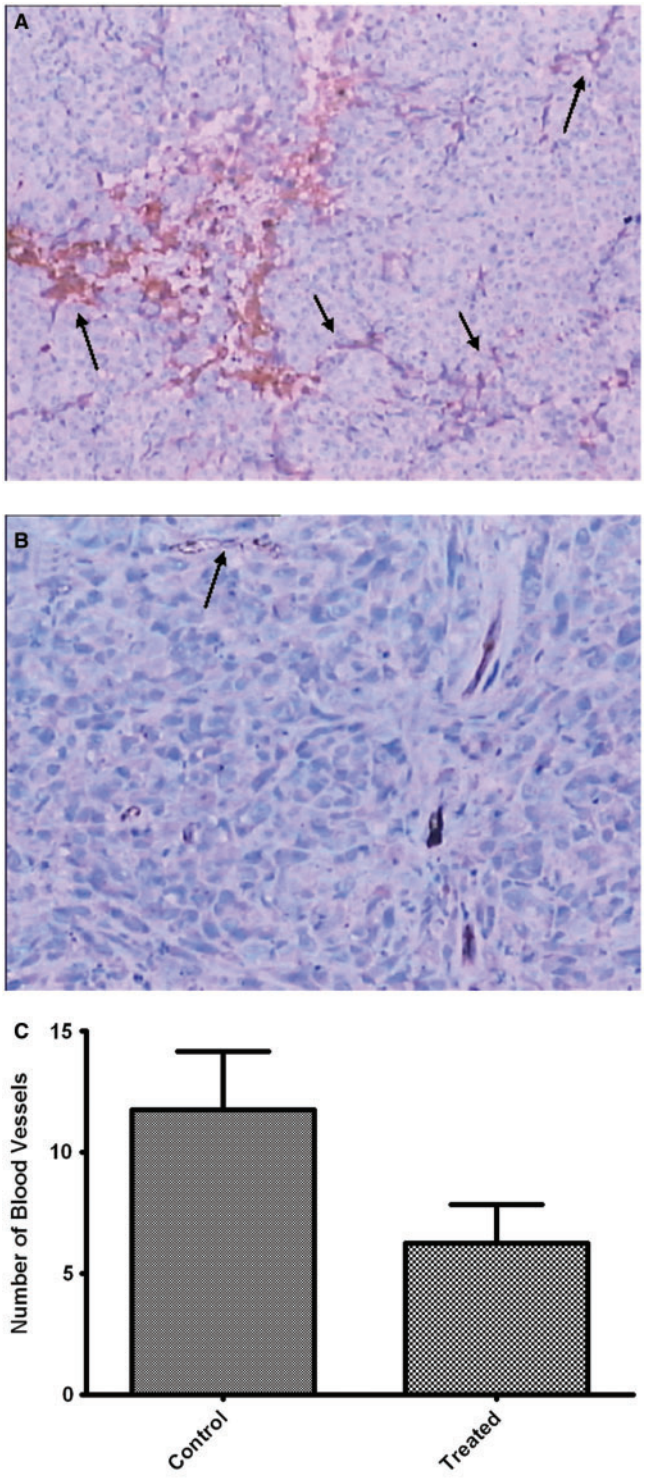

Fig. 4. **Effect of VPA treatment on Tumour Angiogenesis.** Sectioned tumour xenografts were treated with CD31 antibodies, and then counter stained with Haematoxylin and Eosin (HE). As it clearly shows there is significantly more vascularization in control slide (**A**) than that in the treated one (**B**). (**C**) Quantitative estimation of difference of vascularization between control and treated slides from different xenogragrafts. Immunoreactive blood vessels in different sections from all the xenografts were counted. Results here are expressed as means ± SD (*$P < 0.05$).

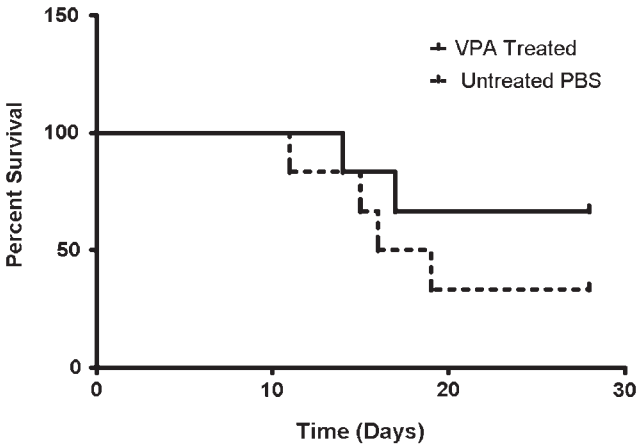

Fig. 5. **Percentage survival of animals in control and treated groups.** Animals in both treated and control groups were monitored every day following inoclution of HeLa cell. By using Kaplan–Meier method, survival times were determined. As shown in the figure, the mean percent survival of VPA treated animals was significantly higher, *i.e*. 66.6% compared to the untreated control which was 33.3% at 28 days.

In our study, we used three cervical cancer cell lines (*i.e*. HeLa, Ca Ski and SiHa) for *in vitro* cell proliferation assay. On the other hand, for the evaluation of effect of VPA treatment on acetylation of histone, expression of p21 and p53, and for the *in vivo* drug studies, we used HeLa cell line only since it is a model of choice among cervical cancers (*28*). VPA was able to inhibit *in vitro* cellular proliferation considerably at doses of 1.2 mM (which is well within therapeutic range) and above. The degree of *in vitro* anti-proliferative effect was more or less consistent in all three cell lines and was dose-dependent across the board. Furthermore, western blot confirmed considerable accumulation of histone H3 at therapeutic concentrations. But histones are not the only molecular targets of HDAC inhibitors, since they can affect cancer cell biology by pathways that do not involve histones. For example, the gene regulatory activity of transcription factors (*e.g*. E2F1, p53, STAT1, STAT3, NF-KB, p21) can be modulated through direct acetylation and deacetylation (*29*). Among these, CDK inhibitor is a key component of cell cycle checkpoints, *i.e*., the G1/S (*30*) and the G2/M checkpoints (*31*, *32*). HDAC inhibitors have previously been reported to either up- (*33*) or down-regulate (*34*) p21WAF1/CIP1 expression in a p53-independent manner in HeLa cell line. Moreover, HDAC inhibitors were shown to induce G1/S phase arrest in cervical carcinoma cells despite p53 and E6/E7 interaction (*35*). In our study, we also evaluated the changes in expression of p21 and p53 after acute VPA treatment *in vitro*. VPA up-regulated p21 gene expression in a dose-dependent manner, while leaving the p53 expression unchanged, irrespective of the investigated concentrations of the drug. Since the induction of cell cycle arrest is known to be associated with an increase in the expression of the cyclin-dependent kinase inhibitor (CDKI) p21/Waf-1, so it would seem logical to deduce that *in vitro* anti-proliferative of VPA is attributed to the cell cycle arrest resulting from p21 up-regulation.

There has been increasing evidence that the use of chronic treatment regimen is more effective, when it comes to VPA (*36–38*). Most of the trails conducted with chronic treatment plan, were designed to maintain a plasma concentration that would guarantee consistent therapeutic level of the drug, while keeping its toxicity in check. We also followed the same pattern and mice, implanted with tumour xenografts, were given VPA in place of drinking water to ensure the consistent serum levels of the drug. Our testing spanned over a period of four weeks, which is normally enough to evaluate survival rate in a given population of mice with tumour implantation. At the end of the trail, we selected one angiogenesis marker, *i.e*. CD31, to assess the tumour response to treatment. VPA has been shown to exhibit a weak anti-angiogenic effect *in vivo* (*8*), yet, we surprisingly found a considerable difference in tumour vasculature in the treated versus untreated controls.

An ulterior motive for employing chronic treatment regimens is to increase the survival time of the animals. We also planned our animal experiments to evaluate the effect of VPA treatment on survival of the animals. We used Kaplan–Meier method to calculate the mean percent survival time in both treatment and control groups. In our study, we discovered that the mice treated with VPA, suffered from comparatively lesser tumour burden and survived longer than that of control group.

In summary, the results from this study demonstrate that VPA is capable of suppressing cervical tumour cell growth both *in vitro* and *in vivo*, and might have a potential therapeutic utility in treating cervical carcinomas.

We are particularly thankful to Wen Hui Jiang and Shuhua Chen of Nanjing Stomatological Hospital, Affiliated Medical School, Nanjing University, Nanjing, for extending their help by providing us the necessary facilities for Immunohistochemical Staining. We are also extremely grateful to Dr Shawn E. Lupold, Assistant Professor at The James Buchanan Brady Urologic Institute, Johns Hopkins University School of Medicine, for helping us with proof reading of the manuscript.

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